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GRAIN INSPECTION, PACKERS AND STOCKYARDS ADMINISTRATION
FEDERAL GRAIN INSPECTION SERVICE
STOP 3630
WASHINGTON, D.C. 20250-3630

CHAPTER 21

NEOGEN NEOCOLUMN TEST METHOD

21.1 GENERAL INFORMATION

The Neogen NeoColumn Aflatoxin DR test method for aflatoxin uses affinity column immunoassay for quantitative measurement of total aflatoxins (B1, B2, G1, and G2) in parts per billion (ppb), or qualitative (screening) for aflatoxin.

21.2 FLUOROMETER CALIBRATION

a. General.

An FGIS-approved fluorometer is used to determine the aflatoxin level. To ensure accurate results, calibrate the fluorometer prior to use each day and verify at least once an hour using the **Yellow Vial**.

Turn the fluorometer on with the On/Off switch located on the rear panel. When the fluorometer is turned on, allow it to warm up for 10 minutes before calibrating. Once the fluorometer is turned on, it may be left on until close of business for the day. If the fluorometer is turned off during the day, a 10-minute warm up is required.

After turning the fluorometer on, it will identify itself and perform a set of self-tests. If any error message appears, consult the operator's manual.

b. Calibration Procedures for Series 4 Fluorometer.

(1) Follow the prompts on the fluorometer display to calibrate the unit. The first time using/setting up the instrument follow the steps below.

(a) Press options key until Test Functions appears and press enter.

(b) Press options key until Create Test appears and press enter.

(c) Using the Alpha numeric keys, name the test (Neocolumn).

(d) When name is complete press enter twice.

(e) When prompted, enter the appropriate calibrator levels for (red, green, and yellow vials).

(f) Choose integer or decimal format for results display and press enter.

(g) Choose ppb as measurements units and press enter.

(h) Set delay to 60 seconds and press enter.

(i) Choose a calibrator expiration time in hours (24 hours expiration limit) and press enter.

Note: This step is applicable to the Series 4 fluorometer only. Calibration values are pre-entered for the DRF 2100 Fluorometer.

(2) Press select test until NeoColumn appears and press enter.

(3) Follow the prompts on the fluorometer display to start calibration sequence.

(4) When prompted to insert a calibration vial, wipe the vial with a clean cloth or paper wipe and insert it into the bottom of the well. Be sure that the vial is fully inserted and touches the bottom of the well.

(5) Insert the red vial and press enter.

(6) Remove the red vial and insert the green vial, and press enter.

(7) Remove the green vial and insert the yellow vial to verify calibration.

(8) Remove yellow vial, and record the results for the Yellow Vial.

(9) If the value of the yellow calibration vial is not within FGIS specifications, repeat the calibration process (steps 2 through 6 listed above), then check the yellow vial again. If the reading for the Yellow Vial remains above or below FGIS specifications, contact the Field Office, TSD, or Neogen Technical Service Division.

(10) When the fluorometer is calibrated, place the standards back in the case and close tightly, and store away from any light source.

(11) Check the calibration of the fluorometer at least once an hour or before analyzing any test samples if more than 1 hour time has elapsed since the last test using the Yellow Vial.

Calibrator values (in ppb) for Corn, Corn Soy Blend, Rough Rice, Milled Rice, and Rice Bran.		
	Series 4	DFR 2100 Afla 0.5 gram calibration
Red	44	44
Green	-1	-1
Yellow	22 (+/- 2)	22 (+/- 2)

c. Calibration procedures for the DRF 2100.

All instructions in the DFR 2100 Operators Manual must be followed for initial set-up and operation of instrument. Technician must adhere to all unpacking, preparation for operation, protocol set-up, and precautions before use.

(1) Operating the DRF 2100.

After the instrument is initialized, the MAIN MENU appears on the display with four options.

(a) Main Menu.

1-Run Assay: This is the option selection for preprogrammed test parameters, calibrating the reader and selecting a user.

2-Stored Results: This option allows the operator to view, print or delete all stored data.

3-Manage protocols: This option allows the operator to view, add new, delete or perform edits to the protocols.

4-Options: This option allows system configuration changes to the time, date, and printer.

(b) Select 1-Run Assay. This screen lists the test programmed into the instrument. The DRF 2100 comes pre-programmed with 2 aflatoxin protocols.

1. AFLA 1.0 GRAM

2. **AFLA 0.5 GRAM for FGIS use**

(c) Select a test from the programmed list. One of the two screens will be displayed: "Calibration: INVALID' or "Calibration: VALID". The validity of the calibration is based on user-inputted reagent data and expiration time information. It is calculated by the instrument, and prompts the user through the appropriate sequence.

(d) Calibration INVALID screen only allows 3 selections available to the user:

1-Run calibration

2-Select User

3-Printer: ON or OFF

(e) Select Run Calibration, and follow all display prompts to calibrate instrument.

Note: The operator must place the correct tube in proper order. The instrument only recognizes that a tube is inserted but not whether it is a red or green tube.

(f) After calibration sequence and receiving 3 audible beeps. The Calibration Valid screen appears and prints. Proceed to the next screen selections yellow vial.

(g) Calibration VALID screen displays the test name and the calibration is valid, and list selections available to the operator.

1-Run Assay

2-Calibrator Check

3-Select User

4-Run calibration

5-More…
1-Sample ID: On/Off

2-Printer: On/Off

(h) Select 1-Run Assay, allows a sample to be analyzed. Follow display instructions then insert a clean cuvette containing sample elution and developer. (See test instructions)

(i) Select Calibrator Check, allows the user to test the Yellow Vial to determine if a sample will be correctly read and the stability of the calibration.

(j) Select User displays SELECT USERNAME with the current user list below. The operator can choose the appropriate name or add new users name to the list. Type in user name using the key pad and press Enter when complete.

(k) Select Run Calibration reverts back to the Calibration Mode. If this is inadvertently selected, the operator can back out of the function using the back◄ key, but once the calibration has begun, it must be completed.

(l) Selecting "More", gives the operator options to enter a sample ID and send information to a printer.

Please read the DRF 2100 operator's manual for more information pertaining to other instrument capabilities.

d. Calibration Standards (NEOGEN Part # 8049).

(1) Maintenance.

The standard solutions in the three (3) standard vials (Red, Green, and Yellow) degrade slowly in the presence of light.

Since the plastic case containing the vials passes a small amount of light, it is recommended that both case and vials be stored in a cabinet or drawer away from all light except when calibrating or checking the calibration of the fluorometer.

Maintain two (2) sets of standards (two cases) at each location. Select and identify one set as the working standard, the other as the reference standard to be used to check the working standard every 14 days.

The degradation of the working set will occur gradually over a period of time, so anticipate expiration and requisition a replacement set in advance. (A sudden change in the reading of a vial indicates instrument instability, a cracked vial, undue exposure of the vial to light, etc.)

When one vial of a set expires, replace the entire set. About 2 months before the expected expiration of the working set, obtain a new set of standards from the Neogen Corporation. When received, compare fluorometer readings of the new set with those of the existing reference set. If the difference between the two sets exceeds 2± ppb for any of the colors, notify TSD.

(2) Biweekly check of working standards.

(a) Calibrate the fluorometer (Series 4 or DRF 2100) using the working set as described in "Calibration Procedures" (see section 21.2 b).

(b) Test the red and green vials from the working set and record the values.

(c) After testing the working set, remove the reference set from storage and test the 3 vials as described in section 21.2, b. The difference in readings of the two sets should not exceed the following limits:

Red	Yellow	Green
± 2 ppb	± 2 ppb (0.5 gram calibration)	± 2 ppb

If the difference between the working and reference sets exceed the tolerances, discard the out of tolerance calibrators. Designate the intolerance set of calibrators as the working set, reorder a new reference set of calibrators. Keep a permanent record of all calibration verification data.

The DRF 2100 does not give digital display values. Instead, calibration will show as valid or invalid. If invalid you must recalibrate. Follow the

steps on the DRF 2100.

21.3 PREPARATION OF SOLUTIONS

Reagents and cuvettes should be checked to assure that there is no background fluorescence. This test should be performed prior to beginning testing procedures, when new reagents are prepared, or when new cuvettes used. The distilled/deionized water, dilute developer solution, and the HPLC grade methanol must be checked for background fluorescence with the fluorometer after properly calibrating. None of the above reagents should give a positive reading of more than 0.0 ppb. Solutions that do not read 0 ppb should be retested using a new cuvette first. If the solution is still not reporting a 0, that solution should be discarded, made fresh and tested again.

a. NeoColumn Developer Solution.

NOTE: Developer Solution must be prepared fresh every 8 hours.

The concentrated developer solution should have a slight reddish brown color. Do not use the stock solution if it is colorless. Loss of color indicates that the stock solution has lost its potency.

Prepare dilute developer solution by adding 5 ml of NeoColumn developer concentrate to 45 ml of distilled/ deionized water. Mix well, and label the dilute developer solution bottle showing the date and time of preparation.

DO NOT USE SOLUTION AFTER 8 HOURS HAVE ELAPSED.

If the amount of dilute developer being prepared needs to be adjusted based on the workload at individual locations, make sure that the 1 part concentrated developer to 9 parts distilled/deionized water ratio is maintained.

Label each stock bottle of concentrated developer with the date on which it was first opened. **DO NOT USE CONCENTRATED DEVELOPER AFTER 30 DAYS HAVE ELAPSED.**

b. 80/20 Percent Methanol Solution.

Make up the solution by using the ratio of 8 parts HPLC or ACS grade methanol to 2 parts deionized/distilled water. Prepare the 80 percent methanol water solution by adding 800 ml methanol to 200 ml of water. Mix well. Keep the bottle tightly capped when not in use.

Label the 80 percent methanol/water solution bottle showing date of preparation. If the amount of the 80 percent methanol solution being prepared needs to be adjusted based on the workload at individual locations, make sure that the 8 parts HPLC grade methanol to 2 parts distilled/deionized water ratio is maintained.

To prepare smaller or larger amounts of solution the ratio of 8 parts methanol to 2 parts of deionized or distilled water must be maintained. For example: To prepare a solution that will provide for 5 test extractions (100 ml per test sample) mix 400 ml HPLC grade methanol to 100 ml deoinized or distilled water.

21.4 SOLUTION TESTING

The distilled/deionized water, NeoColumn developer solution, and HPLC grade methanol must be tested for background fluorescence before use. After calibrating the fluorometer perform the following:

a. Methanol.

Place 2.0 ml of HPLC grade methanol into a clean cuvette. Place the cuvette in the calibrated fluorometer. The displayed reading should be 0 ppb. If the reading is greater than 0 ppb, retest or replace the methanol.

b. Water.

Dispense 2.0 ml of deionized/distilled water into a clean cuvette. Place the cuvette in the calibrated fluorometer. The digital display reading should be 0 ppb. If the reading is greater than 0 ppb, retest or take action to assure a pure water supply.

c. Developer Solution.

Combine 1.0 ml of dilute developer solution and 1.0 ml of HPLC grade methanol in a clean cuvette. Place the cuvette in the calibrated fluorometer. The digital display reading should be 0 ppb.

If the reading is greater than 1.0, check each reagent separately to determine which reagent is causing the problem and replace it.

21.5 TEST PROCEDURES

a. Procedures for Testing Corn, Corn/Soy Blend, Rough Rice, Milled Rice, and Rice Bran.

(1) Extraction.

(a) Place 50 g of ground sample into blender jar.

(b) Add 5 grams of analytical, USP grade sodium chloride (NaCl) or food grade un-iodized salt.

(c) Add 100 ml of the 80/20 methanol/water extraction solution.

(d) Cover jar and blend at high speed for 1 minute.

(e) Remove the cover and pour the extract into a filter paper (Neogen item #9355 fluted filter paper 24 cm, or equivalent) supported in a clean funnel.

(f) Collect the filtrate in a clean beaker labeled with the sample identification.

(g) After collecting approximately 25 ml of extract, carefully dispose of the filter paper and its contents.

(h) Pipette 10 ml of filtered extract into a clean beaker.

(i) Add 40 ml of distilled/deionized water and mix thoroughly.

(j) Filter the diluted extract through a microfibre filter (Neogen part # 9352, or equivalent) supported by a small, clean funnel. Fold the glass microfibre filter gently without making a sharp crease to avoid breaking the glass microfibre filter.

(k) Immediately proceed with the NeoColumn test procedure.

Note: If this diluted filtrate turns cloudy, refilter using a new glass microfibre filter before proceeding with the analysis.

(2) Affinity Column.

(a) Prepare the NeoColumn for use by removing both end caps.

(b) Attach the column to a 10 ml glass syringe barrel in the pump stand.

(c) Place a disposable plastic cup under the column to collect waste.

(d) Using an Eppendorf pipette, or equivalent, add 5.0 ml of the filtered dilute extract to the reservoir.

(e) Apply pressure to the column to initiate flow at a rate on 1–2 drops per second. Ensure no air bubbles form in the column as this will restrict the flow of the sample through the column.

(f) Allow the sample extract to completely pass through the column.

(g) Add 10 ml of deionized or distilled water to the syringe barrel and again apply a steady positive pressure to pass the wash water through the column.

(h) After the wash has passed through the column, place a clean cuvette under the outlet of the column. Use care when handling the cuvette to keep the optical surface clean and free of lint, fingerprints, etc.

(i) Dispense 1.0 ml of HPLC grade methanol into the reservoir.

(j) Apply a steady pressure to elute/pass the methanol through the column and collect all of the methanol eluate in the cuvette. Maintain pressure to collect the methanol at a rate of approximately 1 drop per second.

(k) Add 1.0 ml of dilute NeoColumn Developer Solution directly to the sample eluate solution in the cuvette and mix well (about 5 seconds).

(l) **Immediately** place the cuvette in a calibrated fluorometer.

(3) Reading, Recording, and Certifying Test Results.

(a) Record the digital readout (Series 4) or corresponding value from the printout (DRF-2100) as total ppb.

(b) Report all results on the pan ticket and the inspection log to the nearest whole ppb.

(c) Sample results over 100 ppb are reported as >100 ppb unless a supplemental analysis is performed.

(d) Refer to the Certification section of the handbook for more detailed certification procedures.

(4) Supplemental Analysis.

To determine and report an aflatoxin level higher than 100 ppb, the filtered test sample extract must be diluted so that a value between 5 ppb and 100 ppb is obtained. The final aflatoxin concentration is calculated by multiplying the results obtained with the diluted extract by the dilution factor.

(a) Using an Eppendorf pipette, add 2.5 ml (instead of 5.0 ml) of the filtered diluted extract to the reservoir. (See section 21.5 (2) (d).)

(b) Analyze the filtered extract as a normal sample.

(c) Multiply the analytical results obtained by 2 to obtain the actual aflatoxin concentration. For example, if 90 ppb was the sample value obtained using the diluted test sample procedure, the actual concentration in the original sample was 180 ppb.

Example:	Diluted test sample extract result	90 ppb
	Dilution factor	x 2
	Actual aflatoxin concentration	180 ppb

Note: Laboratories may dilute samples as a first step if levels typically observed exceed 100 ppb and the applicant requests certified results above the range of the test kit.

21.6 CLEANING LABWARE

a. Negative Tests (≤ 20 ppb).

(1) Labware.

Prepare a solution consisting of dishwashing liquid and water. Completely submerge the used glassware, funnels, beakers, etc., wash thoroughly, then rinse with clean water before reusing.

(2) Disposable Materials.

Place materials in a garbage bag for routine trash disposal.

b. Positive Tests (> 20 ppb).

(1) Labware.

Prepare a bleach solution consisting of 1 part bleach to 10 parts water (e.g., 100 ml bleach to 1,000 ml water). Completely submerge the used glassware, funnels, beakers, etc., and soak for at least 5 minutes.

Remove items from the bleach/water solution, submerge in a dishwashing liquid/water solution, wash thoroughly, then rinse with clean water before reusing.

(2) Disposable Materials.

Prepare a bleach solution consisting of 1 part bleach to 10 parts water in a plastic pail labeled "bleach solution." Soak disposable materials, such as used columns, cuvettes, vials, test kit components, etc., for at least 5 minutes. Pour off the liquid down the drain and place the materials in a garbage bag and discard.

21.7 WASTE DISPOSAL

a. Negative Results (≤ 20 ppb).

If the test result is negative (equal to or less than 20 ppb), discard the filter paper and its contents (ground material) into a plastic garbage bag for disposal. Dispose of any remaining liquid filtrate in the chemical waste container.

b. Positive Results (> 20 ppb).

If the result is positive (more than 20 ppb), the ground portion remaining in the filter paper must be decontaminated prior to disposal. After disposing of the remaining filtered extract in the chemical waste container, filter approximately 50 ml of bleach through the filter containing the ground portion and allow to drain. Discard the filter paper and its contents (ground portion) into a plastic garbage bag for disposal. The bleach rinse filtrate collected may be treated as a non-hazardous solution and disposed of by pouring down the drain.

21.8 EQUIPMENT and SUPPLIES

a. Fluorometer -Series 4, or DRF-2100.

b. Amber glass bottle 50 ml (Neogen item # 9496, or equivalent).

c. Amber glass bottle, 500 ml (Neogen item # 9497, or equivalent).

d. Dispenser, 0-3 ml, for 50 ml glass bottle (Neogen item # 9355, or equivalent).

e. Dispenser, 0-3 ml, for 500 ml glass bottle (Neogen item # 9356, or equivalent).

f. Vortex mixer.

g. Blender.

h. Blender jar, stainless steel.

i. Dispenser, 0 – 3 ml, for 500 ml glass bottle (Neogen item #9356, or equivalent).

j. Fluted filter paper, 24 cm (Neogen item # 9351, or equivalent).

k. Microfibre filters 11 cm (Neogen item # 9352, or equivalent).

l. Small plastic funnels.

m. Methanol (HPLC or ACS grade).

n. Disposable cuvettes.

o. Cuvette rack.

p. Disposable plastic cups.

q. Distilled or deionized water.

r. 5 ml pipettor and tips.

s. Glass syringe barrel (10 ml) (Neogen item #965, or equivalent).

t. Pump stand.

u. USP grade sodium chloride (NaCl) or food grade un-iodized salt.

v. 50 ml, 250 ml graduated cylinder.

w. 500 ml wash bottle.

x. NeoColumn Aflatoxin DR calibrators (Neogen item #8049).

y. NeoColumn Aflatoxin DR developer (Neogen item #8048).

z. Laboratory scale.

21.9 STORAGE CONDITIONS

a. Affinity Columns - Store at room temperature (64º to 86º F).

b. Calibration Vials - Store in a cabinet or drawer away from all light, except when in use.

c. Developer Concentrate - Store in a tightly closed bottle in a cool, dry, well ventilated area and away from sunlight, combustible materials, and incompatible materials.